Original article

Multiple infections in questing nymphs and adult female *Ixodes ricinus* ticks collected in a recreational forest in Denmark

Kirstine Klitgaard*, Lene Jung Kjær, Anastasia Isbrand, Mette Frimodt Hansen, Rene Bødker

Department for Diagnostics and Scientific Advice, National Veterinary Institute, Technical University of Denmark, Kgs. Lyngby, Denmark

ABSTRACT

During its lifecycle, the generalist *Ixodes ricinus* takes up three blood meals from a wide selection of vertebrate hosts, some of which are reservoirs for multiple vector-associated pathogens. Since *I. ricinus* also readily bites humans, pets, and livestock, these hosts are at risk of becoming infected with more than one tick-borne pathogen. Multiple tick-borne infections are a public health concern, since they may increase diversity and duration of symptoms and complicate differential diagnosis and therapy. We used an existing Fluidigm real-time PCR chip to identify the minimum risk of exposure to infected/co-infected ticks in Denmark. We screened 509 nymphs and 504 adult female *I. ricinus* ticks for 17 different vector-associated pathogenic agents. The questing ticks were collected by flagging during the same season in two consecutive years in Grib forest in the capital region of Copenhagen. Overall, 19.1% of the nymphs and 52.2% of the adult female ticks harbored at least one zoonotic pathogen. The main agents were *Borrelia* spp., *Anaplasma phagocytophilum* and *Rickettsia helvetica*, while *Candidatus* Neoehrlichia mikurensis and *Babesia venatorum* both were present in less than 1% of the ticks. In 3.5% of the nymphs and 12.3% of adults we found more than one tick-borne pathogen. Of these, 15% were potentially triple or quadruple infections. Whereas mixed infections with *Borrelia* were equally distributed among both life stages, the adult ticks hosted 84.5% of the co-infections with different species of tick-borne pathogens, chiefly involving *Borrelia* species in combination with either *R. helvetica* or *A. phagocytophilum*. Statistical analyses indicated non-random co-occurrence of *Borrelia spielmanii/Borrelia garinii* in both life stages and *B. garinii/Borrelia afzelii* and *B. garinii/Borrelia valaisiana* in the nymphs. Although the overall prevalence of ticks hosting more than one infection only constituted 7.9% at the particular site investigated in this study, our results still underline that co-infections should be considered in diagnosis and treatment of tick-borne diseases in northern Europe.

1. Introduction

Hard ticks (Ixodidae) transmit a variety of pathogens affecting the health of both humans and animals. *Ixodes ricinus*, the dominant tick species in Denmark, has four life cycle stages (egg, larva, nymph, and adult). For every instar, the tick requires a blood meal from a vertebrate host, which for the generalist *I. ricinus* comprises a selection of more than 300 vertebrate species (Herrmann and Gern, 2015). With each feeding, the tick can take up several strains of bacteria, viruses, and parasites, either by consuming a single blood meal from a host animal with multiple infections or by transfer between co-feeding ticks infecting each other through infectious saliva (Piesman and Happ, 2001). Some of these tick-borne pathogens may persist in the ticks by transstadial survival (from larva to nymph to adult ticks), in which case a higher rate of infection is expected for these agents in the adult stage

(Jensen et al., 2017b; Rizzoli et al., 2011). Moreover, some pathogens, such as *Rickettsia* and tick-borne encephalitis (TBE)-complex virus, can be transovarially transmitted to the offspring (Karbowiak and Biernat, 2016; Sprong et al., 2009).

So far, disease-causing agents identified from *I. ricinus* in Denmark comprise members of the genus *Borrelia*, *Anaplasma phagocytophilum*, *Rickettsia helvetica*, *Candidatus* Neoehrlichia mikurensis, *Babesia divergens*, *Babesia microti*, *Babesia venatorum*, *Bartonella henselae*, and TBE-complex virus (Fertner et al., 2012; Fomsgaard et al., 2009; Michelet et al., 2014; Nielsen et al., 2004; Skarphédinsson et al., 2005; Stensvold et al., 2015; Vennestrøm et al., 2008). Specifically, *B. burgdorferi*, *B. garinii* and *B. afzelii* have been isolated from Danish patients with Lyme borreliosis (Lebech, 2002).

The genus *Borrelia* includes species which cause Lyme borreliosis, the most widespread vector-borne human infection in the temperate

---

* Corresponding author at: Kemitorvet, building 202, 2800, Kgs. Lyngby, Denmark.
 E-mail address: kksc@vet.dtu.dk (K. Klitgaard).

northern hemisphere (Rizzoli et al., 2011). The symptoms differ somewhat between the species; *Borrelia* afzelii preferentially causes erythema migrans (EM), and acrodermatitis chronica atrophicans; *Borrelia spielmanii* has also been linked to EM in humans (Maraspin et al., 2006). Lyme arthritis (LA) is most commonly associated with *Borrelia burgdorferi* senso stricto infection and there is a high correlation between neuroborreliosis and infection with *Borrelia* garinii (Rudenko et al., 2011). *Borrelia miyamotoi*, which phylogenetically belongs to the relapsing fever *Borrelia*, causes *Borrelia miyamotoi* disease (BMD) in humans (Telford et al., 2015).

Reports of other tick-borne illnesses besides Lyme borreliosis are rare in Denmark, but they may be under-reported due to the often mild and uncharacteristic symptoms and/or lack of awareness in the healthcare sector (Jensen et al., 2017a; Skarphédinsson et al., 2007; Stuen et al., 2013). In Europe, species from the genera *Babesia* and *Anaplasma* are well-known veterinary pathogens, particularly in domestic livestock (Stuen et al., 2013). Human babesiosis is uncommon and so far, no cases of tick-transferred babesiosis have been reported in Denmark (Jensen et al., 2017a). *A. phagocytophilum* is the cause of human granulocytic anaplasmosis (HGA); a disease which is often subclinical or associated with mild, unspecific influenza-like symptoms, but may cause severe or fatal disease in a subset of patients (Dumler et al., 2007). *R. helvetica* may also cause illness characterized by mild influenza-like symptoms including headache, muscle pain, or rashes (Parola et al., 2005). *Candidatus* N. mikurensis is an emerging zoonotic pathogen. In humans, EM-like rashes and infections caused by this bacterium are primarily seen in immunocompromised patients (Grankvist et al., 2014).

Experimental animal studies suggest that dual infections with some of these tick-borne pathogens can alter disease transmission and reduce or enhance severity of infection among hosts (Holden et al., 2005; Swanson et al., 2006; Thomas et al., 2001). Due to these potential public health implications, it is important to quantify the regional prevalence of tick-acquired co-infections (infections by two or more pathogens of different genera) or mixed infections (multiple infections with pathogens of the same genus) (Wójcik-Fatla et al., 2016). According to molecular evidence from other parts of Europe, multiple infections are common in *I. ricinus* (Moutailler et al., 2016; Raulf et al., 2018; Zając et al., 2017). However, in Denmark, less is known about the risk of acquiring more than one vector-borne pathogen from a tick bite (Skarphédinsson et al., 2007; Vennestrøm et al., 2008). In the present study, we therefore screened 509 individual questing nymphs and 504 questing female adult *I. ricinus* ticks for 17 tick-associated agents of zoonotic concern by using real-time PCR (RT-PCR). Here, we quantify co-infections and multiple infections in Denmark and discuss the potential mechanisms underlying these infections.

2. Materials and methods

2.1. Sample collection

We collected a total of 509 questing nymphs and 504 questing adult female ticks in June 2016 (253 nymphs and 251 adults) and June 2017 (256 nymphs and 253 adults). The ticks were collected by flagging from a 40 x 100 m deciduous area situated near a pasture grazed by a low number of cattle in Grib forest, North Zealand, Denmark (56°01′40.3″N, 12°20′11.0″E). Ticks were stored at −80 °C until use.

2.2. DNA extraction

We washed the ticks for 5 min in 70% ethanol and subsequently 2 × 5 min in sterile water. We used a Maxwell 16 LEV Blood DNA kit (Promega, Madison, Wisconsin, USA) for the extraction procedure, applying a protocol adapted for extraction of DNA from ticks. Briefly, we homogenized the ticks using a TissueLyser II (Qiagen, Hilden, Germany) for 2 × 2.5 min at 25 Hz in a mixture of 75 µl Incubation

buffer (D920) and 75 µl Lysis buffer (MC501) and with three 3-mm Tungsten beads (Qiagen). After a brief centrifugation at $10,000 \times g$ for 60 s (at that point, samples could be stored frozen at -20 °C until use), we added 30 µl Proteinase K and incubated the samples at 56 °C overnight. Next, we added 300 µl Lysis buffer (MC501) and vortexed the samples briefly. We isolated genomic DNA using the Maxwell 16 LEV Blood DNA kit (Promega, Madison, Wisconsin, USA) on a Maxwell®16 Instrument according to the manufacturer's instructions.

2.3. Screening of bacterial and parasitic tick-borne pathogens by qPCR

We used the BioMark real-time PCR system (Fluidigm, San Francisco, California, USA) for high-throughput microfluidic RT-PCR, applying the 192.24 dynamic arrays (Fluidigm). The 23 primer/probe sets used for RT-PCR target all the bacterial and parasitic tick-borne pathogens which have previously been identified in *I. ricinus* from Denmark, as well as some of the most common tick-borne pathogens known to circulate in Europe and two tick species: *B. garinii, B. afzelii, B. spielmanii, B. valaisiana, Borrelia lusitaniae,* and *B. miyamotoi, B. burgdorferi, A. phagocytophilum, Candidatus* N. mikurensis, *R. helvetica, Francisella tularensis, Coxiella burnetii, B. divergens, B. microti, B. canis, B. venatorum, B. henselae,* The spotted fever group (SFG) *rickettsiae* and the genus *Borrelia*. The assay also included primer/probe sets targeting *Ixodes ricinus* and *Dermacentor reticulatus* (Michelet et al., 2014). We pre-amplified the DNA in a final volume of 5 µL containing 3.5 µL TaqMan PreAmp Master Mix,1.2 µL pooled primer mix (except for those targeting tick DNA), and 1.3 µL DNA, with one cycle at 95 °C for 10 min, 14 cycles at 95 °C for 15 s and 4 min at 60 °C. We diluted the pre-amplified DNA 5 × in water before we performed RT-PCR using FAM- and black hole quencher (BHQ1)-labeled TaqMan probes with TaqMan Gene Expression Master Mix in accordance with the manufacturer's instructions (Applied Biosystems, Foster City, California, USA). Thermal cycling conditions were as follows: 50 °C for 2 min, 95 °C for 10 min, 40 cycles at 95 °C for 15 s, and 60 °C for 15 s. Data were acquired on the BioMark RT-PCR system and analyzed using the Fluidigm RT-PCR Analysis software to obtain crossing point (CP) values. CP was set to < 26. We included one negative water control per chip. In one lane, we added *Escherichia coli* DNA plus primers and probes for internal inhibition control (Michelet et al., 2014).

2.4. Statistical analysis

We assessed the prevalence with the exact confidence intervals (based on beta distribution) of the individual microbes in the examined ticks and the statistical interaction between pathogens for each instar and year by a two-sided Fisher's exact test using the FREQ procedure in SAS Enterprise Guide 6.1 for Windows 7 Copyright © 2013 SAS Institute Inc. Cary, NC, USA.

3. Results

3.1. Prevalence of infections among the I. ricinus ticks

In total, 19.1% (97/509) of the nymphs and 52.2% (263/504) of the adult females were PCR-positive for at least one tick-borne pathogen. A PCR run was considered valid when all NTCs were negative, all samples were positive for *I. ricinus* DNA (which served both to identify the tested species and as a DNA extraction control), and all *E. coli* controls were positive (served as internal inhibition controls for each sample). All the ticks were of the species *I. ricinus*. We failed to detect *B. lusitaniae, F. tularensis, C. burnetii, B. divergens, B. microti, B. canis,* and *B. henselae*.

Members of the genus *Borrelia* were the most abundant bacteria in both life stages; 8.6% (44/509) of the nymphs and 32.5% (164/504) of the female adults were PCR-positive. *A. phagocytophilum*-positive PCRs were seen in 6.1% (31/509) of the nymphs and 14.3% (72/504) of the female adults, and *R. helvetica*-positive PCRs in 5.5% (28/509) of the

Table 1

DNA prevalence (%) of the tick-borne pathogens identified from *I. ricinus* ticks collected in Grib forest, June 2016 and 2017.

	Nymphs					Adult Females					Sum (n = 1013)
	2016 (n = 253) PCR-positive samples (%)	95% CI	2017 (n = 256) PCR-positive samples (%)	95% CI	2016 + 2017 (n = 509) PCR-positive samples (%)	2016 (n = 251) PCR-positive samples (%)	95% CI	2017 (n = 253) PCR-positive samples (%)	95% CI	2016 + 2017 (n = 504) PCR-positive samples (%)	
Borrelia[#]	11 (4.4)$^{\alpha,*}$	2.2-7.6	33 (12.9)$^{\alpha,*}$	9.0-17.6	44 (8.6)	57 (22.7)*	17.7-28.4	68 (26.9)*	21.5-32.8	125 (24.8)	169 (16.7)
B. afzelii	0 (0.0)$^{\alpha}$		7 (2.7)$^{\alpha}$	1.1-5.6	7 (1.4)	3 (1.2)	0.2-3.5	11 (4.3)	2.2-7.6	14 (2.8)	21 (2.1)
B. valaisiana	0 (0.0)$^{\alpha}$		10 (3.9)$^{\alpha}$	1.9-7.1	10 (2.0)	3 (1.2)	0.2-3.5	3 (1.2)	0.2-3.4	6 (1.2)	16 (1.6)
B. miyamotoi	0 (0.0)*		0 (0.0)*		0 (0.0)	7 (2.8)*	1.1-5.7	6 (2.4)*	0.9-5.1	13 (2.6)	13 (1.3)
B. burgdorferi	0 (0.0)$^{\alpha}$		10 (3.9)$^{\alpha}$	1.9-7.1	10 (2.0)	4 (1.6)$^{\alpha}$	0.4-4.0	14 (5.5)$^{\alpha}$	3.1-9.1	18 (3.6)	28 (2.8)
B. garinii	2 (0.8)$^{\alpha}$	0.1-2.8	13 (5.1)$^{\alpha}$	2.7-8.5$^{\alpha}$	15 (2.9)	7 (2.8)	1.1-5.7	8 (3.2)	1.4-6.1	15 (3.0)	30 (3.0)
B. spielmanii	1 (0.4)*	0.0-2.2	5 (2.0)*	0.6-4.5	6 (1.2)	25 (10.0)*	6.5-14.4	26 (10.3)*	6.8-14.7	51 (10.1)	57 (5.6)
R. helvetica	9 (3.6)*	1.6-6.6	20 (7.8)	4.5-11.3	29 (5.7)	37 (14.7)*	10.6-19.7	30 (11.9)	8.1-16.5	67 (13.3)	96 (9.6)
A. phagocytophilum	12 (4.7)*	2.5-8.1	19 (7.4)*	4.5-11.3	31 (6.1)	36 (14.3)*	10.3-19.3	36 (14.2)*	10.2-19.2	72 (14.3)	103 (10.2)
Candidatus N. mikurensis	0 (0.0)		0 (0.0)		0 (0.0)	0 (0.0)		2 (0.8)	0.1-2.8	2 (0.4)	2 (0.2)
B. venatorum	0 (0.0)		1 (0.4)	0.0-2.2	1 (0.2)	0 (0.0)		4 (1.6)	0.4-4.0	4 (0.8)	5 (0.5)
Sum	35 (13.8)		118 (46.1)		153 (30.1)	179 (71.3)		208 (82.2)		387 (76.8)	540 (53.3)

* Designates significant differences in prevalence (p < 0.05) between the two different instars in a given year.

$^{\alpha}$ Designates significant differences between the two years in prevalence (p < 0.05) for the same instar.

[#] *Borrelia* species that could only be determined at genus level. CI: Confidence Interval.

nymphs and 13.3% (67/504) of the female adults. *B. venatorum* and *Candidatus* N. mikurensis were present in less than 1% of the ticks and the latter was only identified in adult females. The prevalence of PCR-positive pathogens (CP-values < 26) identified for each year and life stage is detailed in Table 1.

In both years, the genus *Borrelia*, *B. miyamotoi*, *B. spielmanii*, and *A. phagocytophilum* were significantly more abundant (Fisher's exact test, p < 0.05) in the adult females compared to the nymphs (Table 1). The difference in prevalence of *R. helvetica* between life stages was significant in 2016 (Fisher's exact test, p = 0.0001). *B. venatorum* had a very low occurrence and was only found in ticks from 2017 and *Candidatus* N. mikurensis-positive samples were solely seen in the adult females from 2017. Yearly variations in infection prevalence were most apparent in the nymphs, where *B. afzelii*, *B. burgdorferi*, *B. valaisiana*, and *B. garinii* were all significantly more abundant in nymphs collected in 2017 (Fisher's exact test, p < 0.05).

3.2. Co-infections and mixed infections in I. ricinus ticks

The overall infection rate was 2.7 times higher in adults compared to nymphs, but the average number of pathogens detected per infected tick was 1.3 in both life stages. Ticks infected with more than one pathogen constituted 3.5% (18/509) of the nymphs and 12.3% of the adult females (62/504). Among infected ticks, 18.6% of the nymphs (18/97) and 23.6% (62/263) of the adults carried more than one pathogen species. Dual infections constituted 66.7% (12/18) and 90.3% (56/62) in nymphs and adults with more than one pathogen, respectively. The pathogen combinations found in nymphs and adult females are shown in Table 2.

In the nymphs infected with more than one pathogen, we found 27.7% (5/18) potential triple infections and 5.6% (1/18) quadruple infections. In the adult ticks infected with more than one pathogen, 8.1% (5/62) were triple infections and 1.6% (1/62) was quadruple infections. However, due to potential cross reaction between primers (pairwise interactions between *B. burgdorferi* s.s./*B. garinii, B. burgdorferi /B. valaisiana*, and *B. afzelii/B. spielmanii*), triple and quadruple infections are not considered further in this study.

Mixed *Borrelia* infections were almost equally distributed between nymphs (46%, 24/52) and adults (54%, 28/52). Ticks with combined *B. garinii/B. spielmanii* infections occurred more often than expected by chance in all life stages both years (Fisher's exact test; N2016:

p < 0.02, A2016: p < 0.008, N2017: p < 0.0001, A2017: p < 0.005). Likewise, mixed infections with *B. garinii/B. valaisiana* were significantly higher than expected by chance in the nymphs (Fisher's exact test; N2016: p < 0.020, N2017: p < 0.01).

We found 58 ticks infected with pathogens of two different genera. The adult female ticks hosted 84.5% (49/58) of these co-infections and the nymphs 15.5% (9/58) (Table 2). 18 adult female ticks and 7 nymphs were infected with the combination *Borrelia/ R. helvetica. B. burgdorferi/A. phagocytophilum* co-occurred in 20 adult females but in none of the nymphs. Co-infections with *R. helvetica/A. phagocytophilum* were present in two nymphs and six adult females. In the adult ticks, we also observed two ticks with the combination *Candidatus* N. mikurensis/ *Borrelia*, and two ticks with the combination *B. venatorum/ Borrelia*. One female adult hosted the combination *B. venatorum/ R. helvetica*. None of these species combinations occurred more often than expected by chance.

4. Discussion

The purpose of this study was to address the knowledge gap regarding infection and co-infections level of pathogens in individual tick samples from Denmark. So far, Danish studies on this subject have evaluated the prevalence of single genera, either *Borrelia* (Vennestrøm et al., 2008) or *Rickettsia* (Kantsø et al., 2010), or screened the prevalence of multiple pathogens in pools of ticks, most often questing nymphs (Jensen et al., 2017b; Michelet et al., 2014). The few available co-infection studies have primarily targeted different species of *Borrelia* (Skarphédinsson et al., 2007, Vennestrøm et al., 2008). In this investigation, we simultaneously tested 1013 individual questing nymphs and female adults for 17 different vector-associated pathogens. The public Grib forest is located 25 km north of Copenhagen and is representative of the moist deciduous forest areas of Eastern Denmark and receives more than 650,000 visitors per year. The aim was to give a detailed description of multiple infections in both nymph and adult questing ticks in two consecutive years in a typical deciduous forest area in the densely populated Eastern Denmark.

The risk of acquiring multiple infections is determined by the prevalence of tick-borne pathogens within the reservoir host and the ticks at the specific geographic location (Swanson et al., 2006). In Grib forest, 35.5% of the collected ticks were infected with at least one pathogen. In agreement with previous Danish investigations, the major

Table 2
Combinations of pathogens observed in 509 questing *I. ricinus* nymphs (N) and 504 adult female *I. ricinus* ticks (A) collected in Grib forest, June 2016 and 2017.

	Bb spp.[*]		Ba		Bv		Bm		Bbs		Bg		Bs		Rh		Ap		CN.		Bbv		Sum	
	N	A	N	A	N	A	N	A	N	A	N	A	N	A	N	A	N	A	N	A	N	A	N	A
Ba	–	–			1	0	0	0	1	3	3	1	3	4	0	0	0	0	0	1	0	1		
Bv	–	–	1	0			0	0	4	2	3	3	1	0	2	0	0	0	0	0	0	0		
Bm	–	–	0	0	0	0			0	0	0	0	0	1	0	4	0	2	0	0	0	0		
Bbs	–	–	1	3	4	2	0	0			2	4	0	3	2	0	0	1	0	0	0	0		
Bg	–	–	_3_	1	_3_	3	0	0	2	4			6	7	1	1	0	0	0	0	0	0		
Bs	–	–	3	4	1	0	0	1	0	3	_6_	_7_			1	5	0	7	0	1	0	1		
Rh	1	8	0	0	2	0	0	4	2	0	1	1	1	5			2	6	0	0	0	1		
Ap	0	10	0	0	0	0	0	2	0	1	0	0	0	7	2	6			0	0	0	0		
CN.	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0			0	0		
Bbv	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0	1	0	0	0	0				
Sum N	1		8		11		0		9		15		11		6		2		0		0		41	
Sum A		18		10		7		7		13		16		29		17		16		2		3		138

[*] Bb *Borrelia* species that could only be determined at genus level in the statistical analysis, Ba: *B. afzelii*, Bv: *B. valaisiana*, Bm: *B. miyamotoi*, Bbs: *B. burgdorferi.*, Bg: *B. garinii*, Bs: *B. spielmanii*, Rh: *R. helvetica*, Ap: *A. phagocytophilum*, *C*N.: *Candidatus* N. mikurensis, Bbv: *B. venatorum*. Significant (P < 0.05) associations are underlined. *.

tick-borne agents were *Borrelia*, *A. phagocytophilum*, and *R. helvetica* (Jensen et al., 2017b; Michelet et al., 2014). The prevalence of infection in the female adult instar was 2.7 times higher (52.2%) than in the nymphs (19.1%), most likely due to transstadial accumulation in the mature ticks. This increase was mainly caused by *Borrelia* spp., and *A. phagocytophilum*.

4.1. Prevalence of multiple infections in nymphs and adult female I. ricinus

The effects of bacteria interacting in the host can be both synergistic and antagonistic (Read and Taylor, 2001). Animal studies indicate that increased severity and duration of illness are some of the complications which may arise from multiple infections (Belongia, 2002; Thomas et al., 2001). Further problems may arise due to misdiagnosis resulting from symptom overlap (Jensen et al., 2017a), and the interactions of multiple species have important consequences in shaping the evolution of parasite virulence and disease severity (Herrmann et al., 2013).

In Europe, molecular evidence indicates that dual infections occur in < 1% to 22% of *I. ricinus* ticks (Capelli et al., 2012; Swanson et al., 2006; Tomanovic et al., 2010). In Denmark, the few available investigations of multiple infections in individual *I. ricinus* have mainly focused on *Borrelia* spp. In a study from 2008 of ticks also collected in Grib forest, the rate of mixed *Borrelia* infections in nymphs was 36/600 (6%) and they were more common than single infections (Vennestrøm et al., 2008). In the present study, 3.5% of the collected *I. ricinus* nymphs and 12.3% of the adult females were infected with more than one disease agent, constituting, respectively, 18.6% and 23.6% of all the infected nymphs and adult instars. The majority of the multiple infections were dual (85%); and, as in other epidemiological data from *Ixodes* ticks in North America and Europe, *Borrelia* spp. were present in most of the multiple infections (Swanson et al., 2006). The 52 mixed *Borrelia* infections were almost equally distributed between nymphs and adults, whereas the mature life stage hosted 85.5% (49/58) of the multiple infections with tick-borne pathogens of different species. Only co-occurrences of *Borrelia* species were more common than expected by chance.

4.2. Mixed Borrelia infections

The frequency of mixed *Borrelia* infections reported here is a minimum as the applied RT-PCR method has a limited sensitivity in detecting species within *Borrelia*. In 46.2% of the samples, the chip was positive for the *Borrelia* genus but negative for all the six *Borrelia* species included on the chip. Consequently, species prevalence and hence also prevalence of mixed infections of *Borrelia* are most likely

underestimated with the present RT-PCR assay, since it is not possible to achieve the same high sensitivity for *Borrelia* species as for the *Borrelia* genus. In a meta-analysis of European *I. ricinus* ticks, the most frequent combination of *Borrelia* was *B. garinii/B. valaisiana* (Rauter and Hartung, 2005). In the present study, these two species were also significantly positively correlated in the nymphs; this was expected, since mixed infections with *Borrelia* types adapted to the same vertebrate reservoir host, birds in this case, are more likely to cluster (Herrmann et al., 2013).

Conversely, mixed infections of *Borrelia* species specialized on different vertebrate reservoir hosts are predicted to have a negative co-occurrence (Rauter and Hartung, 2005). In this context, the significant positive co-occurrences of *B. garinii/B. spielmanii* and *B. garinii/ B. afzelii* in the nymphal stage are somewhat unexpected (Table 2), considering that *B. garinii* is associated with birds and *B. spielmanii* and *B. afzelii* with rodents (Kurtenbach et al., 2002). Because these nymphs are fed only once as larvae, the most likely origin of the mixed infections is from one host infected with multiple pathogens (Ginsberg, 2008). Others before us have also observed this unexpected correlation between *Borrelia* species with different vertebrate hosts in nymphs. Vennestrøm et al. (2007) detected mixed infections of *B. afzelii/B. garinii* and Strube et al. (2010) identified frequent double-infections with *B. garinii* in combinations with *B. spielmanii*, or *B. afzelii*.

Mixed *Borrelia* infections are found in both vertebrate hosts and the tick vector (Durand et al., 2017; Rauter and Hartung, 2005; Wójcik-Fatla et al., 2016); and human hosts can be infected with multiple species (Demaerschalck et al., 1995; Ruzic-Sabljic et al., 2005; Swanson et al., 2006; Wójcik-Fatla et al., 2016). At least five of the *Borrelia* species detected in this study are considered human pathogens with distinct clinical manifestations of disease. Presently, the literature on the effects of mixed *Borrelia* infections is limited. However, there are examples of dual *Borrelia* infections resulting in a more severe clinical course and confounding diagnosis. In a mouse model, Hovius et al. (2007) found that mixed infections with *B. burgdorferi* and *B. garinii* resulted in more severe Lyme borreliosis. According to relatively recent reports, the incidence of co-infections with BMD and Lyme borreliosis is 14–19% in the United States (Krause et al., 2014). Also, a recent case from Japan show that mixed infection with *B. miyamotoi* and other *Borrelia* species can result in mixed symptoms, which can confuse diagnosis (Oda et al., 2017).

4.3. Co-infections between different species of tick-associated pathogens

Meta-analyses of *I. ricinus*-complex ticks have shown that co-infections with species from genus *Borrelia/A. phagocytophilum* and species

from the genera *Borrelia/Rickettsia* are relatively common and geographically widespread, and generally occur more often than expected by chance (Nieto and Foley, 2009; Raulf et al., 2018). In this study, we did not observe any such significant co-associations between species and genera. While mixed *Borrelia* infections appeared to be equally distributed between the two instars, 84.5% of all co-infections were seen in the adult *I. ricinus*. In a small-scale study from 2007, *Borrelia* and *A. phagocytophilum* occurred together in 1.9% of the Danish *I. ricinus* ticks (2/106) (Skarphédinsson et al., 2007). We found this combination—and almost as many co-infections of *Borrelia/R. helvetica*—in 4% of the adults (8% of the infected adults). The only two genera combinations in the nymphs were *Borrelia /R. helvetica* (1.4% of all nymphs and 7.2% of the infected nymphs) and *R. helvetica/A. phagocytophilum* (0.4% of all nymphs and 2.1% of the infected nymphs). In the adult females, *Rickettsia/A. phagocytophilum* were the least prevalent combination (1.2% of all female adults and 2% of the infected). This is in agreement with a German study including both nymphs and adult stages, where 1% of the ticks were co-infected with *Rickettsia/A. phagocytophilum* (Hildebrandt et al., 2010). In the adult females, we also detected a few co-infections with *Borrelia/ Candidatus* N. mikurensis, *Borrelia/B. venatorum*, and *R. helvetica/B. venatorum*.

There is evidence which suggests that co-infection with *Borrelia* and *A. phagocytophilum* has a major impact on bacterial fitness, transmission, and pathology (Cabezas-Cruz et al., 2018). Dual infections with these pathogens may result in increased bacterial burden, as well as more severe and diverse clinical manifestation of disease, which in turn makes diagnosis challenging (Holden et al., 2005; Krause et al., 2002; Thomas et al., 2001). For example, combined infection with B. burgdorferi and A. phagocytophilum may result in more severe LD-associated arthritis, than single infection with B. burgdorferi alone (Swanson et al., 2006). Mice studies suggest that this is mainly due to the immunosuppressive nature of *A. phagocytophilum* (Holden et al., 2005).

5. Conclusion

In Grib forest approximately 1/5 of the nymphs contained a vector-borne pathogen, and of these a minimum of 18.6% were mixed *Borrelia* infections. In adult females, more than half of all questing ticks carried the risk of a tick-borne infection and almost a quarter of these contained more than one pathogen. Besides mixed *Borrelia* infections, we found a risk of catching co-infections with HGA and LD, which may worsen disease and confound diagnosis. This study showed that co-infections are relatively common and therefore should be considered in diagnosis of tick-borne diseases and that further studies on other Scandinavian locations are needed in future studies.

Funding

References

Belongia, E.A., 2002. Epidemiology and impact of coinfections acquired from *Ixodes* ticks. Vector Borne Zoonotic Dis. 2, 265–273.

Cabezas-Cruz, A., Vayssier-Taussat, M., Greub, G., 2018. Tick-borne pathogen detection: what's new? Microbes Infect. 20, 441–444.

Capelli, G., Ravagnan, S., Montarsi, F., Ciocchetta, S., Cazzin, S., Porcellato, E., Babiker, A.M., Cassini, R., Salviato, A., Cattoli, G., Otranto, D., 2012. Occurrence and identification of risk areas of *Ixodes ricinus*-borne pathogens: a cost-effectiveness analysis in north-eastern Italy. Parasites Vectors 5, 61.

Demaerschalck, I., Ben Messaoud, A., De Kesel, M., Hoyois, B., Lobet, Y., Hoet, P., Bigaignon, G., Bollen, A., Godfroid, E., 1995. Simultaneous presence of different *Borrelia burgdorferi* genospecies in biological fluids of Lyme disease patients. J. Clin. Microbiol. 33, 602–608.

Dumler, J.S., Barat, N.C., Barat, C.E., Bakken, J.S., 2007. Human granulocytic anaplasmosis and macrophage activation. Clin. Infect. Dis. 45, 199–204.

Durand, J., Herrmann, C., Genné, D., Sarr, A., Gern, L., Voordouw, M.J., 2017. Multistrain infections with Lyme borreliosis pathogens in the tick vector. Appl. Environ. Microbiol. 83, e02552–16.

Fertner, M.E., Mølbak, L., Pihl, T.P.B., Fomsgaard, A., Bødker, R., 2012. First detection of tick-borne "*Candidatus* Neoehrlichia mikurensis" in Denmark 2011. Euro Surveill. 17, 20096.

Fomsgaard, A., Christiansen, C.B., Bødker, R., 2009. First identification of tick-borne encephalitis in Denmark outside of Bornholm, August 2009. Euro Surveill. 14, 2–3.

Ginsberg, H.S., 2008. Potential effects of mixed infections in ticks on transmission dynamics of pathogens: comparative analysis of published records. Exp. Appl. Acarol. 46, 29–41.

Grankvist, A., Andersson, P., Mattsson, M., Sender, M., Vaht, K., Höper, L., Sakiniene, E., Trysberg, E., Stenson, M., Fehr, J., Pekova, S., Bogdan, C., Bloemberg, G., Wennerås, C., 2014. Infections with the tick-borne bacterium "*Candidatus* Neoehrlichia mikurensis" mimic noninfectious conditions in patients with B cell malignancies or autoimmune diseases. Clin. Infect. Dis. 58, 1716–1722.

Herrmann, C., Gern, L., 2015. Search for blood or water is influenced by *Borrelia burgdorferi* in *Ixodes ricinus*. Parasites Vectors 8, 4–11.

Herrmann, C., Gern, L., Voordouw, M.J., 2013. Species co-occurrence patterns among Lyme borreliosis pathogens in the tick vector Ixodes ricinus. Appl. Environ. Microbiol. 79, 7273–7280.

Hildebrandt, A., Krämer, A., Sachse, S., Straube, E., 2010. Ticks and tick-borne diseases detection of *Rickettsia* spp. and *Anaplasma phagocytophilum* in *Ixodes ricinus* ticks in a region of Middle Germany (Thuringia). Ticks Tick. Dis. 1, 52–56.

Holden, K., Hodzic, E., Feng, S., Freet, K.J., Lefebvre, R.B., Barthold, S.W., 2005. Coinfection with *Anaplasma phagocytophilum* alters *Borrelia burgdorferi* population distribution in C3H / HeN mice. Infect. Immun. 73, 3440–3444.

Hovius, J.W., Li, X., Ramamoorthi, N., van Dam, A.P., Barthold, S.W., van der Poll, T., Speelman, P., Fikrig, E., 2007. Coinfection with *Borrelia burgdorferi* sensu stricto and *Borrelia garinii* alters the course of murine Lyme borreliosis. FEMS Immunol. Med. Microbiol. 49, 224–234.

Jensen, B.B., Ocias, L.F., Andersen, N.S., Dessau, R.B., Krogfelt, K.A., Skarphedinsson, S., 2017a. Flåtbårne infektioner i Danmark. Ugeskr. Læger 179, 892–896.

Jensen, P.M., Christoffersen, C.S., Moutailler, S., Michelet, L., Klitgaard, K., Bødker, R., 2017b. Transmission differentials for multiple pathogens as inferred from their prevalence in larva, nymph and adult of *Ixodes ricinus* (Acari: Ixodidae). Exp. Appl. Acarol. 71, 171–182.

Kantsø, B., Svendsen, C.B., Jensen, P.M., Vennestrøm, J., Krogfelt, K.A., 2010. Ticks and tick-borne diseases seasonal and habitat variation in the prevalence of *Rickettsia helvetica* in *Ixodes ricinus* ticks from Denmark. Ticks Tick. Dis. 1, 101–103.

Karbowiak, G., Biernat, B., 2016. The role of particular tick developmental stages in the circulation of tick-borne pathogens affecting humans in Central Europe. 2. Tick-borne encephalitis virus. Ann. Parasitol. 62, 3–9.

Krause, P.J., Mckay, K., Thompson, C.A., Sikand, V.K., Lentz, R., Lepore, T., Closter, L., Christianson, D., Telford, S.R., Persing, D., Radolf, J.D., Spielman, A., 2002. Disease-specific diagnosis of coinfecting tickborne zoonoses: Babesiosis, human granulocytic ehrlichiosis, and Lyme disease. Clin. Infect. Dis. 34, 1184–1191.

Krause, P.J., Narasimhan, S., Wormser, G.P., Barbour, A.G., Platonov, A.E., Brancato, J., Lepore, T., Dardick, K., Mamula, M., Rollend, L., Steeves, T.K., Diuk-Wasser, M., Usmani-Brown, S., Williamson, P., Sarksyan, D.S., Fikrig, E., Fish, D., the Tick Borne Diseases Group, 2014. *Borrelia miyamotoi* sensu lato seroreactivity and seroprevalence in the Northeastern United States. Emerg. Infect. Dis. 20, 1183–1190.

Kurtenbach, K., De Michelis, S., Etti, S., Schäfer, S.M., Sewell, H., Brade, V., Kraiczy, P., 2002. Host association of *Borrelia burgdorferi* sensu lato – the key role of host complement. Trends Microbiol. 10, 74–79.

Lebech, A.M., 2002. Polymerase chain reaction in diagnosis of Borrelia burgdorferi infection and studies on taxonomic classification. APMIS 110 (Suppl. 105), 1–40.

Maraspin, V., Ruzic-sabljic, E., Strle, F., 2006. Lyme borreliosis and Borrelia spielmanii. Emerg. Infect. *Dis.* 12, 1177.

Michelet, L., Delannoy, S., Devillers, E., Umhang, G., Aspan, A., Juremalm, M., Chirico, J., van der Wal, F., Sprong, H., Pihl, T.P.B., Klitgaard, K., Bødker, R., Fach, P., Moutailler, S., 2014. High-throughput screening of tick-borne pathogens in Europe. Front. Cell. Infect. Microbiol. 4, 103.

Moutailler, S., Valiente Moro, C., Vaumourin, E., Michelet, L., Tran, F.H., Devillers, E., Cosson, J.-F., Gasqui, P., Van, V.T., Mavingui, P., Vourc'h, G., Vayssier-Taussat, M., 2016. Co-infection of ticks: the rule rather than the exception. PLoS Negl. Trop. Dis. 10, 1–17.

Nielsen, H., Fournier, P.-E., Pedersen, I.S., Krarup, H., Ejlertsen, T., Raoult, D., 2004. Serological and molecular evidence of *Rickettsia helvetica* in Denmark. Scand. J. Infect. Dis. 36, 559–563.

Nieto, N.C., Foley, J.E., 2009. Meta-analysis of coinfection and coexposure with *Borrelia burgdorferi* and *Anaplasma phagocytophilum* in humans, domestic animals, wildlife, and *Ixodes ricinus*-complex ticks. Vector-borne Zoonotic Dis. 9, 93–101.

Oda, R., Kutsuna, S., Sekikawa, Y., Hongo, I., Sato, K., Ohnishi, M., Kawabata, H., 2017. The first case of imported *Borrelia miyamotoi* disease concurrent with Lyme disease. J. Infect. Chemother. 23, 333–335.

Parola, P., Paddock, C.D., Raoult, D., 2005. Tick-borne rickettsioses around the world: emerging diseases challenging old concepts. Clin. Microbiol. Rev. 18, 719–756.

Piesman, J., Happ, C.M., 2001. The efficacy of co-feeding as a means of maintaining *Borrelia burgdorferi* : a North American model system. J. Vector Ecol. 26, 216–220.

Raulf, M., Jordan, D., Fingerle, V., Strube, C., 2018. Association of *Borrelia* and *Rickettsia* spp. and bacterial loads in *Ixodes ricinus* ticks. Ticks Tick. Dis. 9, 18–24.

Rauter, C., Hartung, T., 2005. Prevalence of *Borrelia burgdorferi* sensu lato genospecies in *Ixodes ricinus* ticks in Europe: a metaanalysis. Appl. Environ. Microbiol. 71,

7203–7216.

Read, A.F., Taylor, L.H., 2001. The ecology of genetically diverse infections. Ecol. Evol. Infect. 292, 1099–1103.

Rizzoli, A., Hauffe, H.C., Carpi, G., Vourc, G.I., Neteler, M., Rosà, R., 2011. Lyme borreliosis in Europe. Euro Surveill. 16, pii 19906.

Rudenko, N., Golovchenko, M., Grubhoffer, L., Oliver Jr, J.H., 2011. Ticks and tick-borne diseases updates on *Borrelia burgdorferi* sensu lato complex with respect to public health. Ticks Tick. Dis. 2, 123–128.

Ruzic-Sabljic, E., Arnez, M., Logar, M., Maraspin, V., Lotric-Furlan, S., Cimperman, J., Strle, F., 2005. Comparison of *Borrelia burgdorferi* sensu lato strains isolated from specimens obtained simultaneously from two different sites of infection in individual patients. J. Clin. Microbiol. 43, 2194–2200.

Skarphédinsson, S., Jensen, P.M., Kristiansen, K., 2005. Survey of tickborne infections in Denmark. Emerg. Infect. Dis. 11, 1055–1061.

Skarphédinsson, S., Lyholm, B.F., Ljungberg, M., Søgaard, P., Kolmos, H.J., Nielsen, L.P., 2007. Detection and identification of *Anaplasma phagocytophilum*, *Borrelia burgdorferi*, and *Rickettsia helvetica* in Danish *Ixodes ricinus* ticks. APMIS 115, 225–230.

Sprong, H., Wielinga, P.R., Fonville, M., Reusken, C., Brandenburg, A.H., Borgsteede, F., Gaasenbeek, C., van der Giessen, J.W.B., 2009. *Ixodes ricinus* ticks are reservoir hosts for *Rickettsia helvetica* and potentially carry flea-borne *Rickettsia* species. Parasites Vectors 2, 41.

Stensvold, C.R., Al Marai, D., Andersen, L.O., Krogfelt, K.A., Jensen, J.S., Larsen, K.S., Nielsen, H.V., 2015. *Babesia* spp. and other pathogens in ticks recovered from domestic dogs in Denmark. Parasites Vectors 8, 262.

Strube, C., Montenegro, V.M., Epe, C., Eckelt, E., Schnieder, T., 2010. Establishment of a minor groove binder-probe based quantitative real time PCR to detect *Borrelia burgdorferi* sensu lato and differentiation of *Borrelia spielmanii* by osp A-specific conventional PCR. Parasites Vectors 3, 69.

Stuen, S., Granquist, E.G., Silaghi, C., 2013. *Anaplasma phagocytophilum* — a widespread multi-host pathogen with highly adaptive strategies. Front. Cell. Infect. Microbiol. 3, 1–33.

Swanson, S.J., Neitzel, D., Reed, K.D., Belongia, E.A., 2006. Coinfections acquired from *Ixodes* ticks. Clin. Microbiol. Rev. 19, 708–727.

Telford III, S.R., Goethert, H.K., Molloy, P., Berardi, V., Chowdri, H.R., Gugliotta, J.L., Lepore, T.J., 2015. *Borrelia miyamotoi* disease (BMD): neither Lyme disease nor relapsing fever. Clin. Lab. Med. 35, 867–882.

Thomas, V., Anguita, J., Barthold, S.W., 2001. Coinfection with *Borrelia burgdorferi* and the agent of human granulocytic ehrlichiosis alters murine immune responses, pathogen burden, and severity of lyme arthritis. Infect. Immun. 69, 3359–3371.

Tomanovic, S., Radulovic, T., Masuzawa, T., Milutinovic, M., 2010. Coexistence of emerging bacterial pathogens in *Ixodes ricinus* ticks in Serbia. Parasite 17, 211–217.

Vennestrøm, J., Egholm, H., Jensen, P.M., 2008. Occurrence of multiple infections with different *Borrelia burgdorferi* genospecies in Danish *Ixodes ricinus* nymphs. Parasitol. Int. 57, 32–35.

Wójcik-Fatla, A., Zając, V., Sawczyn, A., Sroka, J., Cisak, E., Dutkiewicz, J., 2016. Infections and mixed infections with the selected species of *Borrelia burgdorferi* sensu lato complex in *Ixodes ricinus* ticks collected in eastern Poland: a significant increase in the course of 5 years. Exp. Appl. Acarol. 68, 197–212.

Zając, V., Wójcik-Fatla, A., Sawczyn, A., Cisak, E., Sroka, J., Kloc, A., Zając, Z., Buczek, A., Dutkiewicz, J., Bartosik, K., 2017. Prevalence of infections and co-infections with 6 pathogens in *Dermacentor reticulatus* ticks collected in eastern Poland. Ann. Agric. Environ. Med. 24, 26–32.